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MiR-185 is involved in human breast carcinogenesis by targeting *Vegfa*

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ABSTRACT

***MiR-185* expression has been associated with many cancers. However, the roles of *miR-185* in human breast cancer remain elusive. Here, we found that *miR-185* expression was decreased in human breast cancer tissues compared with healthy tissue controls. Up-regulation of *miR-185* inhibited breast cancer cell proliferation and invasion and vice versa. *MiR-185* was shown to bind to the 3′-untranslated region (UTR) of vascular endothelial growth factor a (*Vegfa*), and a significant inverse correlation was found between *miR-185* and *Vegfa*. *Vegfa* overexpression partially restored the inhibition of cell proliferation and invasion that was induced by *miR-185*, and vice versa. Additionally, *Vegfa* expression was found to be high in human breast cancer tissues. Thus, miR-185-mediated Vegfa targeting may be involved in breast cancer formation.**

1. Introduction

MicroRNAs (miRNAs) are small non-protein-coding RNAs that negatively regulate gene expression by inhibiting the translation or reducing the stability of target mRNAs [1]. Many miRNAs are differentially expressed in breast cancers tissues as compared to adjacent normal breast tissues by high-throughput miRNA expression profiling [2–4]. For example, *miR-124* was dramatically down-regulated in breast cancer tissues in human [5]. The proliferative and invasive ability in ERα-positive and ERα-negative breast cancer cells were inhibited by *miR-196a2** expression and enhanced by *miR-196a2** antagonism [6].

MiR-185 takes part in a series of cellular activities, such as cell proliferation and tumorigenesis [7–9]. In recent years, increasing studies mainly focus on its possible roles in tumorigenesis. Xiang et al. found that *miR-185* was markedly down-regulated in the cisplatin-resistant ovarian cancer cells. Moreover, the up-regulation of *miR-185* increased the sensitivity of ovarian cancer cells to cisplatin-induced apoptosis via altering the DMNT1 expression [7]. In poor survival and metastasis colorectal cancer, *miR-185* was observably up-regulated [8]. Ectopic expression of *miR-185* could suppress the growth of human non small cell lung cancer cell lines and induce G1 cell cycle arrest in H1229 cells [9]. However, little is known about the pathophysiological significance of *miR-185* in breast cancer.

In order to investigate the molecular mechanisms by which *miR-185* may execute in breast cancer, its target genes were searched. TargetScan and miRanda prediction algorithms revealed many possible *miR-185* targets. Among them, we found that there was a highly conserved *miR-185* responsive element in 3′-untranslated region (3′-UTR) of *Vegfa*. It was reported that *Vegfa* was a key regulator in angiogenesis and highly expressed in tumor tissues. Furthermore, it closely associates with tumor aggressive features [10,11]. Serum VEGFA concentrations had been elevated in patients with locoregional ductal cancers compared with women with benign breast cancer. The patients with metastatic breast tumor would receive the highest concentrations of serum VEGFA, in particular among patients who did not obtain cancer therapy [12]. Thus, we speculate that *Vegfa* may be the target gene of *miR-185*.

Abbreviations: miRNAs, MicroRNAs; *Vegfa*, vascular endothelial growth factor a; 3′-UTR, 3′-untranslated region; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase; FITC, fluorescein isothiocyanate; PI, phosphatidylinositol; PVDF, polyvinylidene fluoride membrane; HRP, horseradish peroxidase; ER, estrogen receptor; PR, progesterone receptor

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In this study, the expression pattern of *miR-185* and its target gene *Vegfa* was evaluated in breast cancer tissues and adjoining normal breast tissues. The pathophysiological effects of *miR-185* in breast cancer were researched via in vitro cell model. Our study reveals that the *miR-185* may be a novel biomarker for the diagnosis and therapy to human breast cancer in the future.

2. Materials and methods

2.1. Tissue samples and breast cell lines

Human breast cancer tumor tissues and adjacent normal tissues were acquired from the Third Affiliated Hospital of Harbin Medical University (Harbin, China). Table 1 summarized the clinicopathologic characteristics of patients with breast tumors. Before initiation of the study, we obtained the approval of the Ethics Committee of National Research Institute for Family Planning (No. 2011–12) and informed consent from patients. Sixty pairs of human breast cancer tissues and adjoining normal tissues were collected. Partial tissues were fixed with 4% paraformaldehyde solution (Sigma–Aldrich) for *in situ* detection, and partial tissues were frozen in liquid nitrogen for RNA and protein analysis.

Human breast carcinoma cell lines T47D, MCF-7, MDA-MB-231 and MDA-MB-453 were acquired from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). These cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 10 mg/ml streptomycin. All cells were maintained at 37 °C and 5% carbon dioxide in a humidified atmosphere.

Table 1
Clinicopathologic characteristics of patients and breast tumors.

Clinical parameters	Cases (n = 60)	Control (n = 60)
ER PR HER2		
ER(+)/PR(+)/HER2(+)	14 (60)	14 (60)
ER(−)/PR(−)/HER2(−)	15 (60)	15 (60)
ER(+)/PR(+)/HER2(−)	19 (60)	19 (60)
ER(−)/PR(−)/HER2(+)	12 (60)	12 (60)
Tumor size (cm)		
⩽2	26 (60)	26 (60)
>2	34 (60)	34 (60)
No. of positive lymph nodes		
0	29 (60)	29 (60)
1–3	13 (60)	13 (60)
>3	18 (60)	18 (60)
TNM stage		
I	9 (60)	9 (60)
II	39 (60)	39 (60)
II–III	5 (60)	5 (60)
III	7 (60)	7 (60)

2.2. Plasmid construction and transfection

The *Vegfa* 3′-UTR were amplified from human genomic DNA by PCR using the primers in Table 2. The PCR product was double digested with *Spe* I and *Pst* I, and then cloned into pGL3 control vector (designated as Vegfa-pGL3). Mutating the binding sites of *miR-185* in the 3′-UTR of *Vegfa* was used as control (designated as Vegfa-pGL3-Mutant). The VEGFA transcript was amplified from total mRNA of human T47D cells by RT-PCR using the primers in Table 2. The PCR product was double digested with *Xba* I and *Sac* I, and then cloned into PCAGGS-IRES-EGFP (PCA) control vector (designated PCA-Vegfa). All the constructs were verified by DNA sequencing.

Pre-miR control, *miR-185* mimic, anti-miR control, *miR-185* inhibitor, scramble siRNA control and *Vegfa* siRNA were synthesized by GenePharma (GenePharma). The sequences for scramble and *Vegfa* siRNA were as follows: scramble siRNA, UUCUCCGAACGUGUCACGU-dTdT, *Vegfa* siRNA, Sense: 5′-GGAGUACCCUGA UGAGAUCdTdT-3′, antisense: 5′-GAUCUCAUCAGGGUA CUCCdTdT-3′.

2.3. Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR)

Total RNAs were extracted from human breast tissues or cells using Trizol reagent (Invitrogen). The expression of *miR-185* was detected by TaqMan miRNA RT-Real Time PCR. Total RNAs were reverse-transcribed by TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). The acquired single-stranded cDNA were amplified by TaqMan Universal PCR Master Mix (Applied Biosystems) and miRNA-specific TaqMan MGB probes: *miR-185* and *U6* (Applied Biosystems). The U6 was used for internal control. The expression of *Stmn1* was detected by RT-Real-time PCR. Total RNAs were reverse-transcribed by iScriptTM cDNA Synthesis Kit (Bio-Rad). The single-stranded cDNA were amplified by a FastStart Universal SYBR Green Master (Roche) by the primers in Table 2. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used an endogenous control. Each sample in each group was detected in triplicate and the experiment was repeated three times.

2.4. Cell proliferation assay

In vitro cell viability was analyzed by MTS assay. MCF-7 and T47D were transfected with *miR-185* mimic and pre-miR control, respectively. MDA-MB-231and MDA-MB-453 was transfected with *miR-185* inhibitor or anti-miR control. After 48 h of transfection,

Table 2
Primer sequences.

Gene name	Primer sequence	Accession number	Size and location	Application
Vegfa 3′-UTR (sense)	Forward/SpeI: 5′-GGACTAGTGATGTATCTTTTGCTCTCTCTTGCTC-3′ Reverse/PstI: 5′-AACTGCAGCTGCACTAGAGACAAAGACGTGATG-3′	NM-005563	444 bp (636–1079)	PCR
VEGF-pGL3-mutant1	Forward: 5′-TAAAATTCATGTTTCCAATCTCTATATAACTGATCGGTGACAGTCACTAGC-3′ Reverse: 5′-TTATATAGAGATTGGAAACATGAATTTTATATACAAAAACCGGTACAAAT-3′	NM-005563	276 bp (636–911)	PCR
VEGF-pGL3-mutant2	Forward: 5′-AGAATTCTACATACTAAATCTATATAATTTTTTAATTTTAATATTTG-3′ Reverse: 5′-TTATATAGATTTAGTATGTAGAATTCTCTATTTTTTCTTGTTTTGTT-3′	NM-005563	161 bp (919–1079)	PCR
VEGFA transcript	Forward/XbaI: 5′-GCTCTAGAGCATGAACTTTCTGCTGTCTTGGGTGC-3′ Reverse/XhoII: 5′-CCCTCGAGGTGCACTAGAGACAAAGACGTGATG-3′	NM-005563.3	450 bp (177–626)	RT-PCR
Vegfa	Forward: 5′-TTTCTGCTGTCTTGGGTGCATTGG-3′ Reverse: 5′-ACCACTTCGTGATGATTCTGCCCT-3′	NM-005563	131 bp (461–611)	qRT-PCR
gapdh	Forward:5′-GTAGAGGCAGGGATGATGTTC-3′ Reverse: 5′-TGGTATCGTGGAAGGACTCA-3′	NM-001100	129 bp (1270–1386)	qRT-PCR

20 μl MTT (5 mg/ml; Sigma–Aldrich) was added into each well and incubated for 4 h. The media was removed and each well was wash three times by phosphate buffered saline (PBS). 150 μl dimethyl sulfoxide (Sigma–Aldrich) was added into each well and the absorbance was measured by a 96-well plate reader (Bio-Rad 3550) at a wavelength of 570 nm. There were triplicate wells in each treatment group. The experiment was repeated for three times. The cell proliferation capacity was represented by the ratio of absorbance with *miR-185* mimc or *miR-185* inhibitor vs its corresponding control.

2.5. Flow cytometry analysis

Cells apoptosis was detected by flow cytometry analysis using Annexin V-FLUOS staining kit (Roche, Mannheim, Germany). Cells suspension were added with 2.5 μl annexin V-fluorescein isothiocyanate (FITC) stock and 5 μl 20 μg/ml phosphatidylinositol (PI). The mixture was incubated 15 min at room temperature in the dark. The early and late apoptotic populations were analyzed by flow cytometry (BD Biosciences). Each sample acquired 8000 events. Each treatment was detected in duplicative and the experiment was repeated for three times.

2.6. In vitro migration and invasion assays

MCF-7 and T47D were transfected with *miR-185* mimic or pre-miR control, respectively. MDA-MB-231 and MDA-MB-453 was transfected with *miR-185* inhibitor or anti-miR control, respectively. After 48 h of transfection, the transfected cells were harvested and subjected to the following assays. For migration assays, the transfected cells (0.5×106 cells/ml) were seeded in the top of an 8.0-μm-pore membrane chamber (Corning). Cells were fixed by 4% paraformaldehyde solution (Sigma–Aldrich) and stained with hematoxylin and eosin (Sigma–Aldrich) after 17 h of incubation. For invasion assays, matrigel (BD Biosciences) was added in the top of an 8.0-μm-pore membrane chamber (Corning) and incubated at room temperature until the matrigel solidified. Cells (0.5×106 cells/ml) were seeded on the top of matrigel-coated membrane. Cells were fixed by 4% paraformaldehyde solution (Sigma–Aldrich) and stained with hematoxylin and eosin (Sigma–Aldrich) after 24 h of incubation. Cells through membrane were counted under a light microscope. The amount of cells passing through the membrane from five different fields per sample at 200× selected in a random manner was used to determine the capacity of cell migration or invasion.

2.7. Dual-luciferase activity assay

T47D cells were seeded in 48-well plates at a density of 1×10^5, and then transfected by lipofectamine 2000 (Invitrogen) according to the manufacture's instruction. pRL-TK containing Renilla luciferase was used for normalization of gene expression in transiently transfected cells. After 48 h of transfection, cells were harvested. The luciferase activity was analyzed by the dual-luciferase kit (Promega). Each treatment was detected in triplicate and the experiment was repeated for three times. The results were expressed as relative luciferase activity (Firefly LUC/Renilla LUC).

2.8. Western blot

The extracted protein samples from human breast tissues or cells (40 μg) were separated on 12% sodium dodecylsulfate (SDS) polyacrylamide gels and electrotransferred onto polyvinylidene fluoride membrane (PVDF) (Amersham Pharmacia Biotech). The membrane was incubated with primary antibodies anti-VEGFA (Cell Signaling Technology) or anti-GAPDH (Santa Cruz Biotechnology Inc.) overnight at 4 °C. Then the membrane was incubated with horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, Inc.). The bands were visualized with the ECL kit (Millipore). GAPDH was used as an internal control. The experiment was repeated at least three times.

2.9. Statistical analysis

All statistical analyses were performed using SPSS 16.0. All values are reported as the mean ± S.E.M. One-way analysis of variance (ANOVA) was used to perform multiple group comparisons. A linear correlation assay was performed using Pearson correlation. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. The expression profiles of miR-185 in human breast cancer tissues

To investigate the role of *miR-185* in the occurrence of human breast cancer, we analyzed the expression profiles of *miR-185* in 60 pairs of human breast cancer tissues and adjoining normal breast tissues by qRT-PCR (Table 1 and Fig. 1). In order to reduce individual difference of the samples, we pooled cancer tissues form two patients with similar clinical characteristics as a sample (30 samples for 60 pairs). The expression level of *miR-185* was dramatically reduced in 70% (21/30 samples) of breast cancer tissues (Fig. 1A). When compared with compared with adjoining normal tissues, *miR-185* level was obviously diminished in all human breast cancer tissues ($P < 0.01$; Fig. 1B).

3.2. MiR-185 regulates breast cancer cells proliferation in vitro

In order to figure out the possible functions of *miR-185* in the pathological progression of breast cells, we detected the effect of *miR-185* on the growth of breast cancer cells. To choose appropriate cell lines to execute over-expression and knockdown of *miR-185*, we firstly detected the endogenous *miR-185* level in MCF-7,

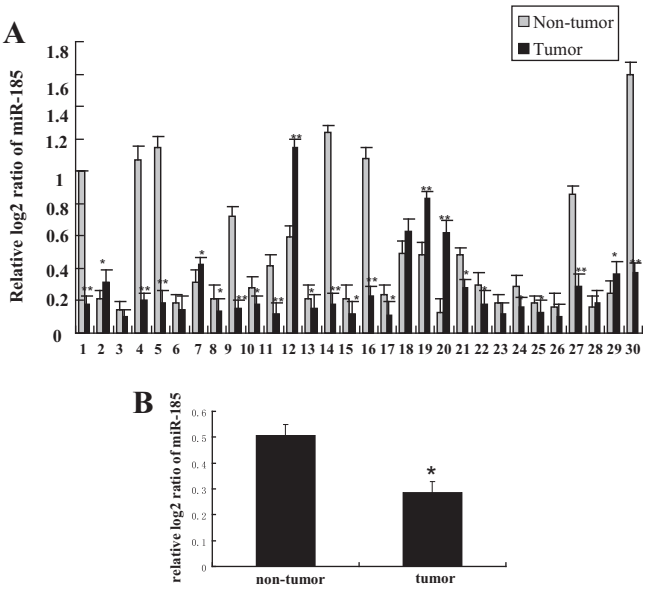

Fig. 1. Down-regulation of *miR-185* in human breast cancer tissues. The cancer tissues from two patients with similar clinical parameters were pooled as a sample. *MiR-185* level in individual sample was detected by TaqMan miRNA RT-Real Time PCR (A). The general trend of *miR-185* in all human breast cancer tissues was analyzed by one-way ANOVA (B). The relative level of *miR-185* was normalized to U6. $^*P < 0.05$.

T47D, MDA-MB-453and MDA-MB-231 cells by qRT-PCR. The *miR-185* level in MDA-MB-231 was higher than that in MCF-7 ($P < 0.01$) and T47D ($P < 0.01$), but approximate with that in MDA-MB-453 (Fig. S1). After that, the effect of *miR-185* mimic or inhibitor on *miR-185* expression in these cancer cell lines was detected by qRT-PCR. Compared with pre-miR control, *miR-185* mimic markedly enhanced *miR-185* level ($P < 0.01$), and *miR-185* inhibitor obviously reduced *miR-185* expression compared with pre-miR control (Fig. S2; $P < 0.01$).

The cell proliferation in *miR-185* mimic or inhibitor-treated cells was detected by MTT assay after 48 h of transfection (Fig. 2). Compared with pre-miR control, the relative proliferation rates in *miR-185* mimic-treated MCF-7 and T47D cells were respectively decreased about 15.95% and 13.75% ($P < 0.05$). However, the relative proliferation rates in *miR-185* inhibitor-treated MDA-MB-231 and MDA-MB-453 cells were respectively increased about 21.82% and 17.5% ($P < 0.05$). All these facts show that over-expression of *miR-185* can inhibit the proliferation of breast cancer cells, while low expression of *miR-185* obviously facilitates breast cancer cells viability.

3.3. MiR-185 affects breast cancer cells apoptosis in vitro

Cell apoptosis in four breast cancer cells was detected by flow cytometry in order to further investigate the role of *miR-185* in regulating the growth of breast cancer cells (Fig. 3). Compared with pre-miR control, the number of early apoptotic cells were enhanced by *miR-185* mimic in T47D (3.43% vs 1.62%), MCF-7 (13.94% vs 9.43%). The number of late apoptotic cells was augmented in T47D (4.57% vs 1.60%), and there was not significant different in MCF-7 cells. Compared with anti-miR control, *miR-185* inhibitor reduced the number of early apoptotic cells in MDA-MB-231 (12.53% vs 17.22%) and MDA-MB-453 cells (5.25% vs 9.84%). The number of late apoptotic cells had no obvious difference. These results indicate that over-expression of *miR-185* can promote cell apoptosis and low expression of *miR-185* inhibits cell apoptosis at early stage.

3.4. MiR-185 regulates the capacity of breast cancer cells migration and invasion in vitro

The influence of *miR-185* on migration and invasion of breast cancer cells were detected in order to further explore the role of *miR-185* in modulating the of breast cancer cells metastasis (Figs. 4 and 5). Compared with pre-miR control, *miR-185* mimic dramatically decreased the capacity of cell migration in T47D and MCF-7 cells ($P < 0.05$). The migration capacity was significantly higher in *miR-185* inhibitor-treated MDA-MB-231 and MDA-MB-453 cells than corresponding control (Fig. 4; $P < 0.05$). Furthermore, the capacity of invasion assay revealed that *miR-185* mimic significantly inhibited the invasive capacity of MCF-7 and T47D cells compared with pre-miR control ($P < 0.05$). However, the invasive ability was obviously increased in *miR-185* inhibitor-treated MDA-MB-231 and MDA-MB-453 cells (Fig. 5; $P < 0.05$). These results indicate that the expression level of *miR-185* may be closely related with breast cancer cells metastasis.

3.5. Vegfa is a target gene of miR-185

To confirm whether *Vegfa* is target gene of *miR-185*, the dual-luciferase assay was used to analyze the relationship between miR-185 and human *Vegfa* 3′-UTR fragment containing the binding sites of *miR-185* (Fig. 6A). Vegfa-pGL3 and *miR-185* mimic or inhibitor were co-transfected into to T47D cells (Fig. 6B). The luciferase activity was decreased about 44.19% by *miR-185* mimic ($P < 0.05$) compared with the pre-miR control. Additionally, the luciferase activity was increased about 88.34% by *miR-185* inhibitor when compared with the anti-miR control, ($P < 0.01$). These findings

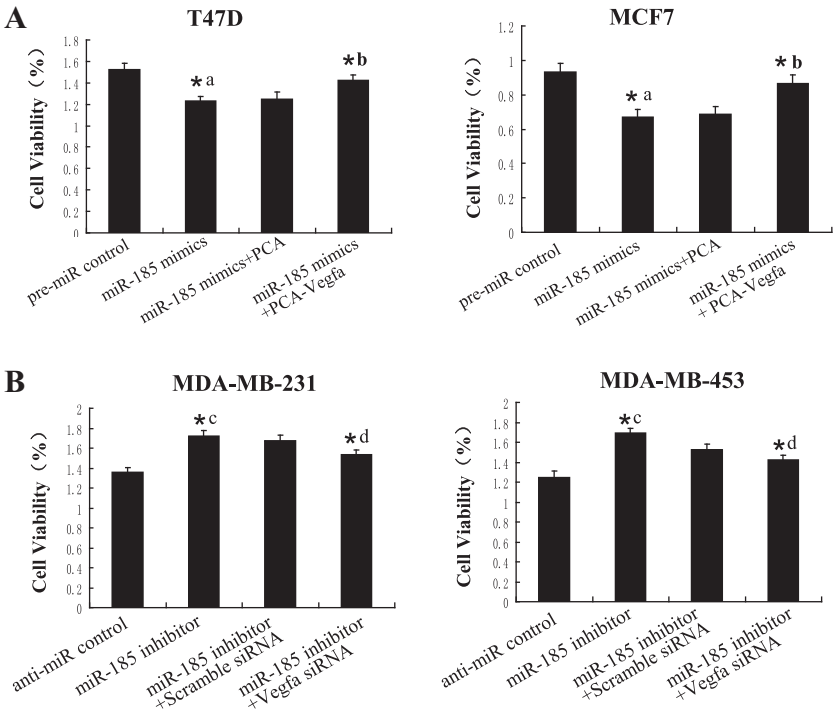

Fig. 2. Cell proliferation in breast cancer cells. *MiR-185* mimic and/or PCA-Vegfa or empty vector control PCA were/was respectively transfected into MCF-7 or T47D (A). *MiR-185* inhibitor and/or *Vegfa* siRNA or scramble siRNA control were/was respectively transfected into MDA-MB-231 or MDA-MB-453 (B). After 48 h of transfection, cell proliferation was determined by MTT assay. All experiments were performed at least three times. *a: *miR-185* mimic vs pre-miR control; *b: *miR-185* mimic + PCA-Vegfa vs *miR-185* mimic or *miR-185* mimic + PCA; *c: *miR-185* inhibitor vs anti-miR control; *d: *miR-185* inhibitor + *Vegfa* siRNA vs *miR-185* inhibitor vs *miR-185* inhibitor + Scramble siRNA. *$P < 0.05$.

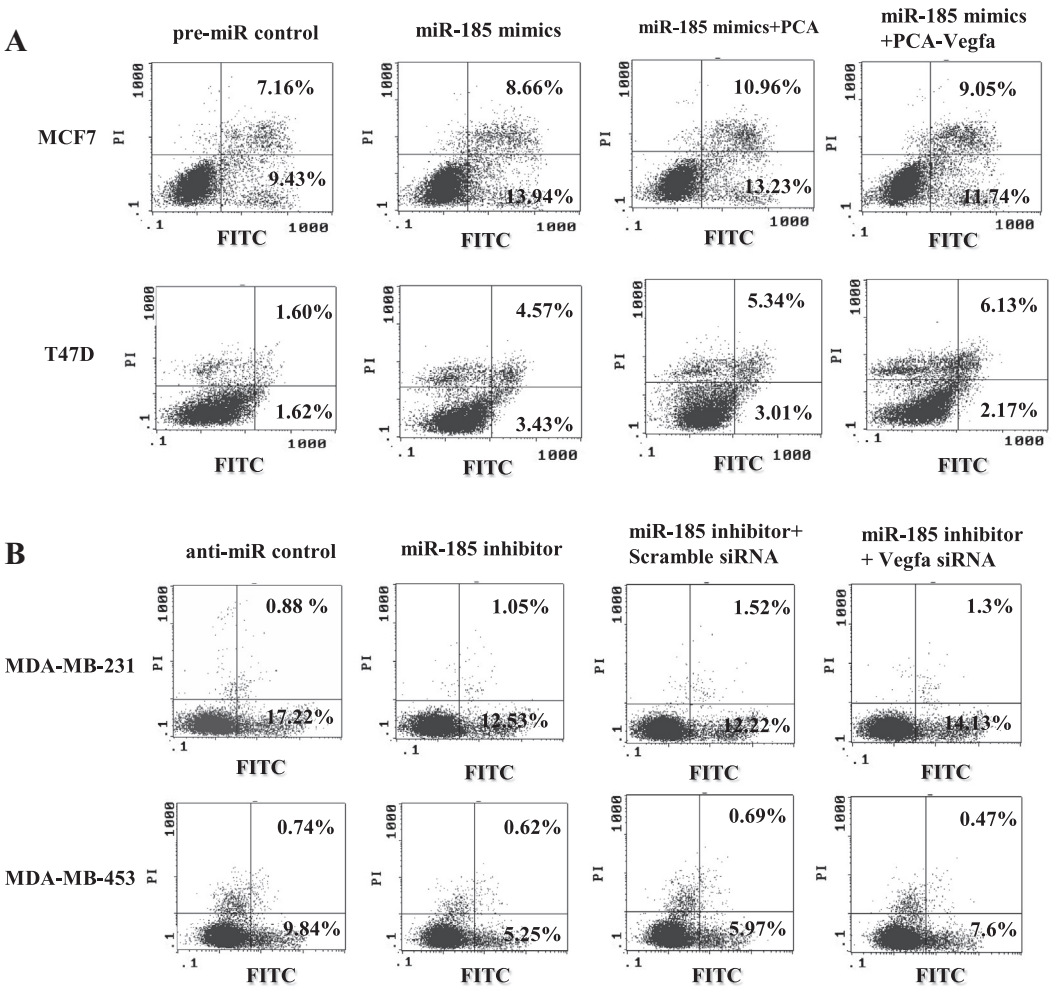

Fig. 3. Apoptosis in breast cancer cells. *MiR-185* mimic and/or PCA-Vegfa or empty vector control PCA were/was transfected into MCF-7 or T47D (A), and *miR-185* inhibitor and/or *Vegfa* siRNA or scramble siRNA control were/was transfected into MDA-MB-231 or MDA-MB-453 (B). After 48 h of transfection, single cell suspension was prepared and stained with annexin V/PI and subjected to flow cytometry analysis. Lower left quadrant, viable cells (annexin V-FITC and PI negative); lower right quadrant, early apoptotic cells (annexin V-FITC positive and PI negative); upper right quadrant, late apoptosis/necrosis cells (annexin V-FITC and PI positive). The percentage of early and late apoptotic cells (representatives of three separate experiments) was shown in the lower right and upper right panels, respectively.

show that *miR-185* has effects on the binding ability of *miR-185* and 3′-UTR of *Vegfa*.

Afterwards, we conducted base mutation of seed sequence to further validate the binding sites for *miR-185* (Fig. 6C). PGL3 empty vector and Vegfa-pGL3-Mutant that mutated the *miR-185* binding sites in the 3′-UTR of *Vegfa* were used as control. Compared with Vegfa-pGL3-Mutant or pGL3 empty vector, the enzyme activity was significantly decreased in cells co-transfected with *miR-185* mimic and Vegfa-pGL3 ($P < 0.05$). These results show that *Vegfa* may be a direct target gene of *miR-185* that inhibits *Vegfa* expression through binding to seed sequence in *Vegfa* 3′-UTR.

3.6. MiR-185 affects the expression of Vegfa in breast cancer cells in vitro

T47D cells were transfected with *miR-185* mimic or inhibitor to analyze the effects of *miR-185* expression disorder on endogenous VEGFA expression. VEGFA protein level was significantly down-regulated by *miR-185* mimic compared with pre-miR control ($P < 0.05$), while *miR-185* inhibitor markedly promoted VEGFA expression ($P < 0.05$) (Fig. 6D). Furthermore, qRT-PCR was used to detect *Vegfa* mRNA level. *miR-185* mimic significantly decreased *Vegfa* mRNA level ($P < 0.05$), while *miR-185* inhibitor markedly increased *Vegfa* mRNA level ($P < 0.05$) (Fig. 6E). These findings show that the mRNA and protein level of endogenous *Vegfa* can be regulated by *miR-185*.

3.7. Vegfa is involved in the influence of mir-185 on cell viability and metastasis

We assessed the influences of *Vegfa* on *miR-185*-mediated cell growth and metastasis in order to further validate whether *miR-185* executed tumor-suppressive functions by targeting *Vegfa*. The influence of *Vegfa* constructs on *Vegfa* expression was detected by qRT-PCR and Western blot. The mRNA and protein level of *Vegfa* was obviously increased by PCA-Vegfa ($P < 0.01$) and markedly reduced by *Vegfa* siRNA (Fig. S3; $P < 0.01$). Co-transfection of PCA-Vegfa and *miR-185* mimic increased the ability of proliferation, migration and invasion and decreased the level of apoptosis compared with *miR-185* mimic alone in T47D and MCF7 cells (Figs. 2A, 3A, 4A and 5A), implying that the up-regulation of *Vegfa* could partially rehabilitate the inhibition of cell proliferation and metastasis mediated by *miR-185* overexpression. Additionally, Co-transfection of *Vegfa* siRNA and *miR-185* inhibitor reduced cell proliferation, migration and invasion ability and enhanced cell apoptosis level compared *miR-185* inhibitor alone in MDA-MB-

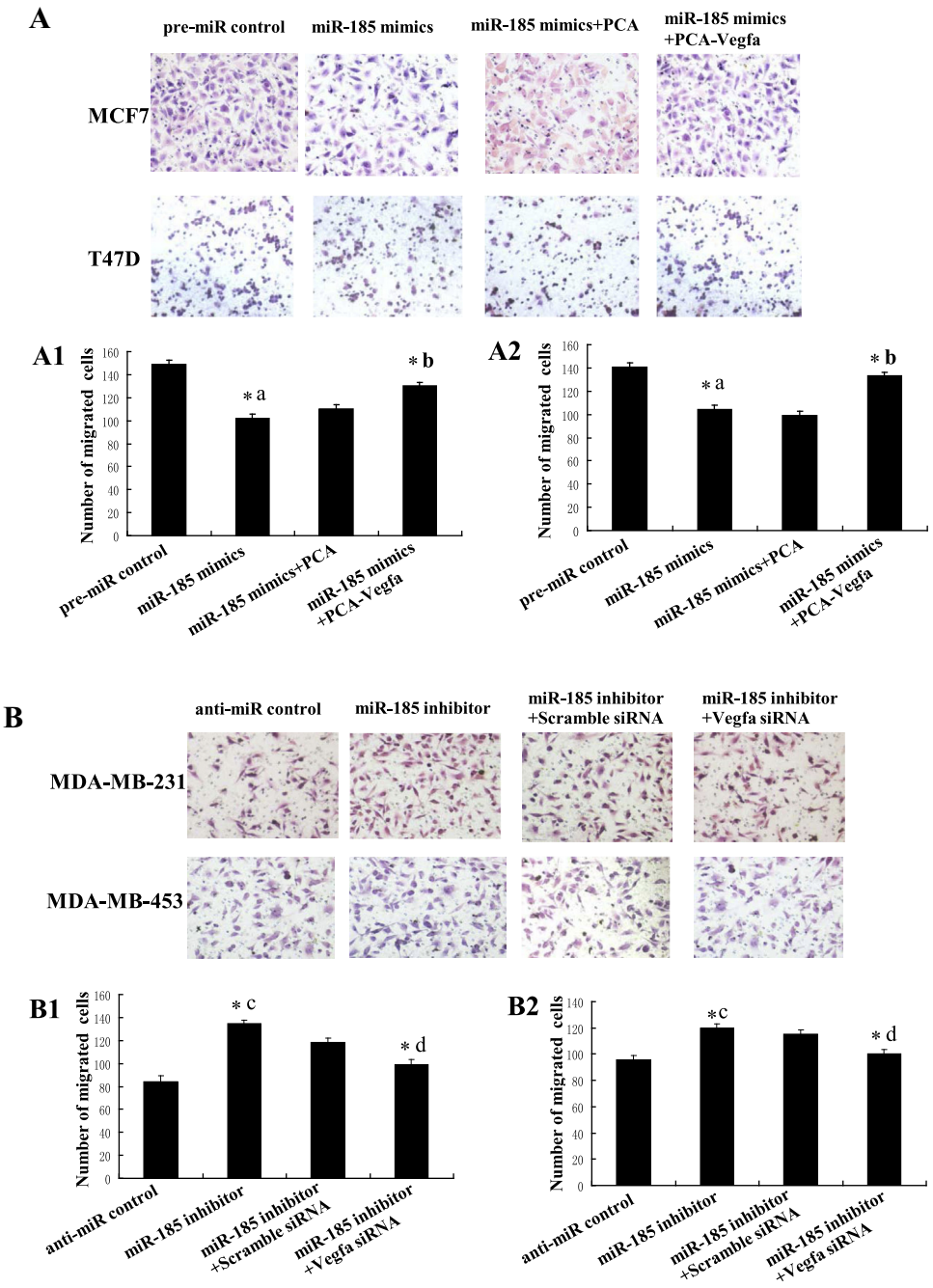

Fig. 4. Cell migration in breast cancer cells. Cells migratory capacity was detected in MCF-7 or T47D cells transfected by *miR-185* mimic and/or PCA-Vegfa or empty vector control PCA (A), and in MDA-MB-231 or MDA-MB-453 transfected by *miR-185* inhibitor and/or *Vegfa* siRNA or scramble siRNA control (B). Cells that had migrated to the filter bottom were quantified by counting the amount of cells passing through the membrane from five different fields per sample at 200× selected in a random manner. A and B show the representative photomicrographs of cells passing through the membrane at 200× original magnification. Histogram A1, A2, B1 and B2 represent the number of migrated cells in MCF-7, T47D, MDA-MB-231 and MDA-MB-453 cells, respectively. Data are expressed as the mean numbers of independent triplicate experiments. *a: *miR-185* mimic vs pre-miR control; *b: *miR-185* mimic + PCA-Vegfa vs *miR-185* mimic or *miR-185* mimic + PCA; *c: *miR-185* inhibitor vs anti-miR control; *d: *miR-185* inhibitor + *Vegfa* siRNA vs *miR-185* inhibitor vs *miR-185* inhibitor + Scramble siRNA. *$P < 0.05$.

231 and MDA-MB-435 cells (Figs. 2B, 3B, 4B and 5B), displaying that *miR-185* knockdown-mediated the facilitation of cell viability and metastasis was partly attenuated by down-regulation of *Vegfa*. These findings reveal that *miR-185* carries out functions in breast cancer cells partly by regulating *Vegfa*.

3.8. Vegfa expression in human breast cancer tissues

We detected the protein and mRNA level of *Vegfa* in breast cancer tissues by Western blot and qRT-PCR in order to further investigate the expression pattern of *miR-185* target gene *Vegfa* in vivo, (Fig. 7). The protein level of VEGFA was dramatically enhanced in breast cancer tissues (Fig. 7A; $P < 0.05$). Additionally, *Vegfa* mRNA level was obviously augmented in 80% (24/30 samples) of breast cancer tissues (Fig. 7B). When compared with that in adjacent normal breast tissues, the general trend of *Vegfa* in all human breast cancer tissues was increased (Fig. 7B1; $P < 0.05$). The expression profiles of *Vegfa* is inverse with *miR-185* in breast cancer tissues, suggesting that *miR-185* may take part in the occurrence of breast cancer by targeting *Vegfa* in vivo.

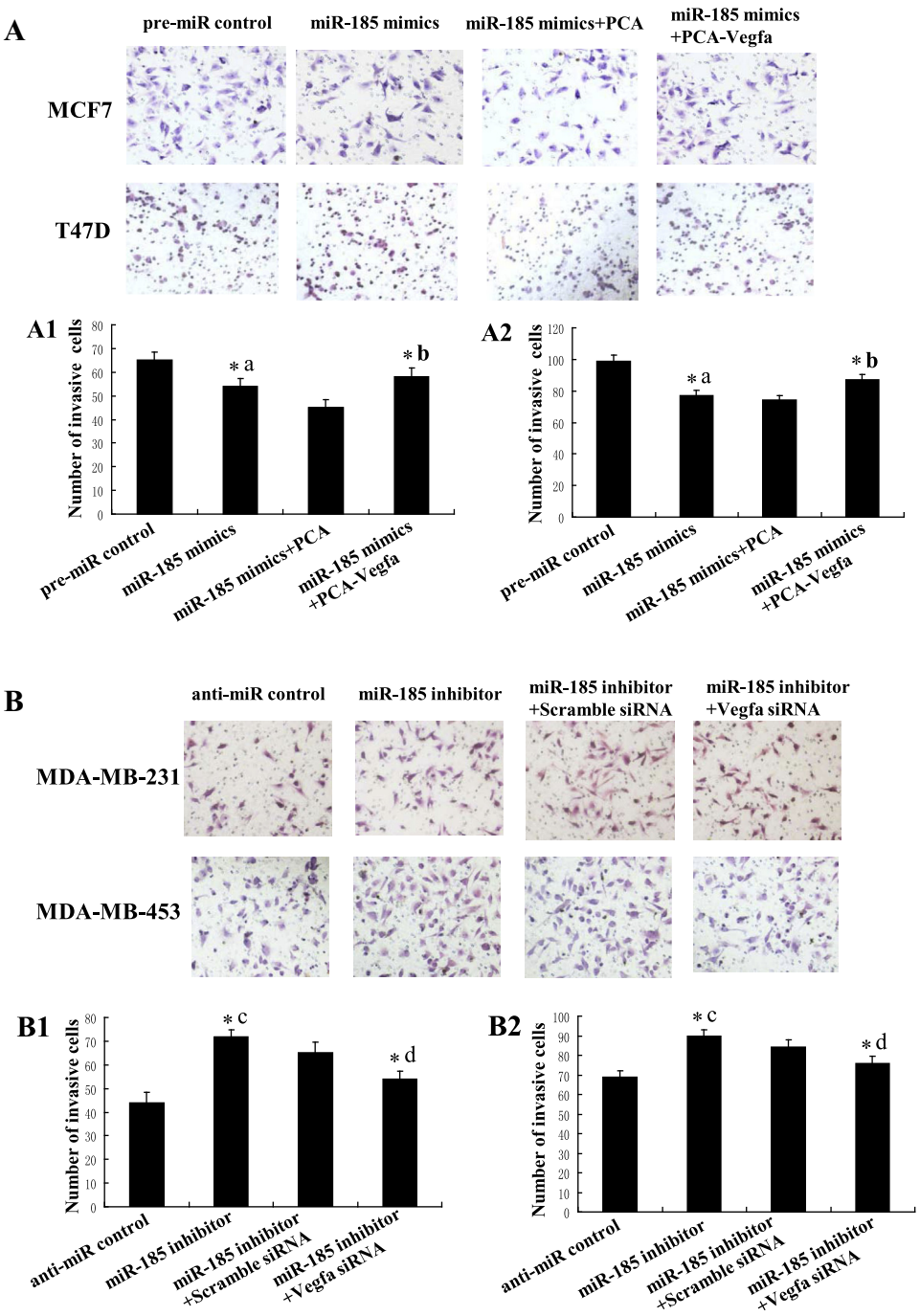

Fig. 5. Cell invasion in breast cancer cells. Cells invasive capacity was detected in MCF-7 or T47D cells transfected by *miR-185* mimic and/or PCA-Vegfa or empty vector control PCA (A), and in MDA-MB-231 or MDA-MB-453 transfected by *miR-185* inhibitor and/or *Vegfa* siRNA or scramble siRNA control (B). Cells that had migrated to the filter bottom were quantified by counting the amount of cells passing through the membrane from five different fields per sample at 200× selected in a random manner. A and B show the representative photomicrographs of cells passing through the membrane at 200× original magnification. Histogram A1, A2, B1 and B2 represent the number of invaded cells in MCF-7, T47D, MDA-MB-231 and MDA-MB-453 cells, respectively. Data are expressed as the mean numbers of independent triplicate experiments. *a: *miR-185* mimic vs pre-miR control; *b: *miR-185* mimic + PCA-Vegfa vs *miR-185* mimic or *miR-185* mimic + PCA; *c: *miR-185* inhibitor vs anti-miR control; *d: *miR-185* inhibitor + *Vegfa* siRNA vs *miR-185* inhibitor vs *miR-185* inhibitor + Scramble siRNA. *$P < 0.05$.

3.9. The correlation between miR-185 and Vegfa in human breast cancer tissues

In order to further demonstrate the relationship between *miR-185* and *Vegfa*, a linear correlation assay was carried out by SPSS16.0. Different housekeeping genes were used as control for normalization of *miR-185* and *Vegfa* expression. In order to decrease experiment error, we adopted the ratio of *miR-185* or *Vegfa* in breast cancer tissues *vs* adjacent normal breast tissues to execute the linear correlation assay. A significant inverse correlation was found between *miR-185* and *Vegfa* on human breast cancer tissues (Fig. 8; $P < 0.01$). The Pearson Correlation Coefficient is −0.573.

4. Discussion

In this study, we provided the first evidence of the association of *miR-185* and breast cancer, although some studies had showed that

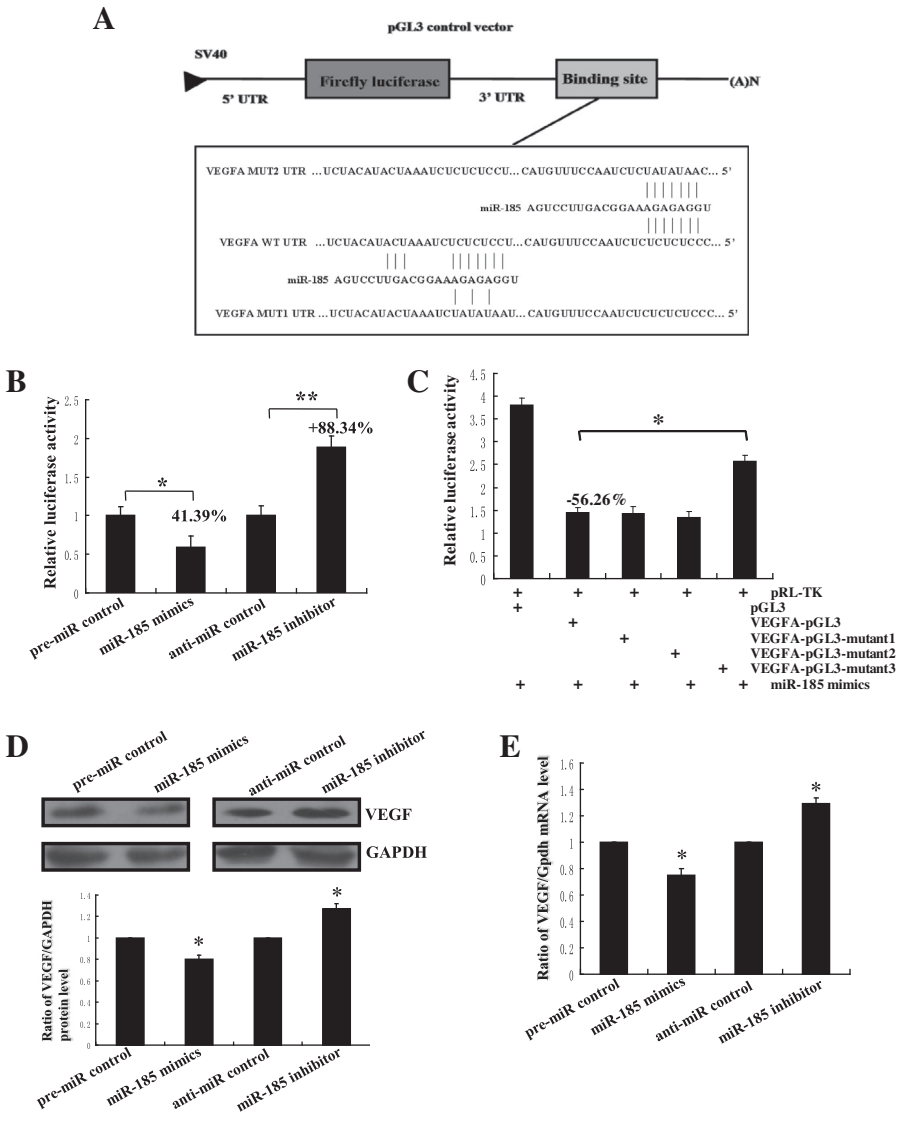

Fig. 6. The prediction and confirmation of the *miR-185* target gene. (A) Schematic diagram for constructing the *miR-185* binding site into pGL3 control vector. (B) Confirmation of the target gene of *miR-185*. T47D cells were co-transfected with pre-miR control, *miR-185* mimic, anti-miR control or *miR-185* inhibitor and Vegfa-pGL3 for dual-luciferase assay. (C) Mutation analysis of the *miR-185* binding site. T47D cells were co-transfected with Vegfa-pGL3 or Vegfa-pGL3-Mutant and *miR-185* mimic for dual-luciferase assay. pRL-TK containing Renilla luciferase was co-transfected with Vegfa-pGL3 or Vegfa-pGL3-Mutant for data normalization. (D) VEGFA protein level in *miR-185* mimic or inhibitor-treated T47D cells was detected by Western blot. The bands were analyzed using the Quantity One analyzing system (Bio-Rad). GAPDH is served as an internal control. The black histogram represents the optical densities of the signals quantified by densitometric analysis. (E) The expression of *Vegfa* mRNA in *miR-185* mimic and inhibitor-treated T47D cells were detected by qRT-PCR. *Gapdh* is served as an internal reference. $^{*}P < 0.05$.

miR-185 participated in ovarian cancer, colorectal cancer, non small cell lung cancer and so on [7–9,13,14].

In this study, we found that *miR-185* was dramatically increased in breast cancers tissues when compared with that in adjoining normal tissues. It suggests that *miR-185* may take part in breast carcinogenesis.

We further explore the roles of *miR-185* in the occurrence of breast cancer. Over-expression of *miR-185* in human breast cancer cell lines accelerated apoptosis and suppressed cells proliferation, invasion and migration, and vice versa. It had been reported that overexpression of *miR-185* in ovarian cells could inhibit the growth of cells and promote cellular apoptosis [7]. The differential expression of *miR-185* inhibited the growth of non-small lung cancer cells, lead to the arrest of cell cycle [9]. The roles *miR-185* in breast cancer are similar with that in other cancers. All of these results imply that *miR-185* may act as tumor suppressor in the occurrence of cancer.

It is generally accepted that miRNAs carry out their function by modulating the expression of their target genes. *Vegfa* is a member of the *Vegf* family, which is the most critical driver of vascular formation and required to initiate the formation of immature vessels in tumor [15–17]. The expression of *Vegf* was up-regulated in human breast tumors tissues and negatively influenced survival [10,11]. Overexpression of *Vegf* dramatically reduced tumor uptake and enhanced tumor growth in a murine model of breast cancer [16]. *Vegf* could prevent apoptosis induced by serum starvation and induce expression of the anti-apoptotic proteins Bcl-2 and A1 [18,19]. All these facts indicate that *Vegfa* may be closely related with the occurrence of breast tumor.

In this study, we explored the target genes of *miR-185* and found a conservative seed sequence in the 3′-UTR of *Vegfa* predicted by miRanda and Targetscan. *MiR-185* mimic and inhibitor regulated *Vegfa* 3′-UTR-mediated luciferase activity. Mutating seed sequence in the 3′-UTR of *Vegfa* obviously suppressed the binding

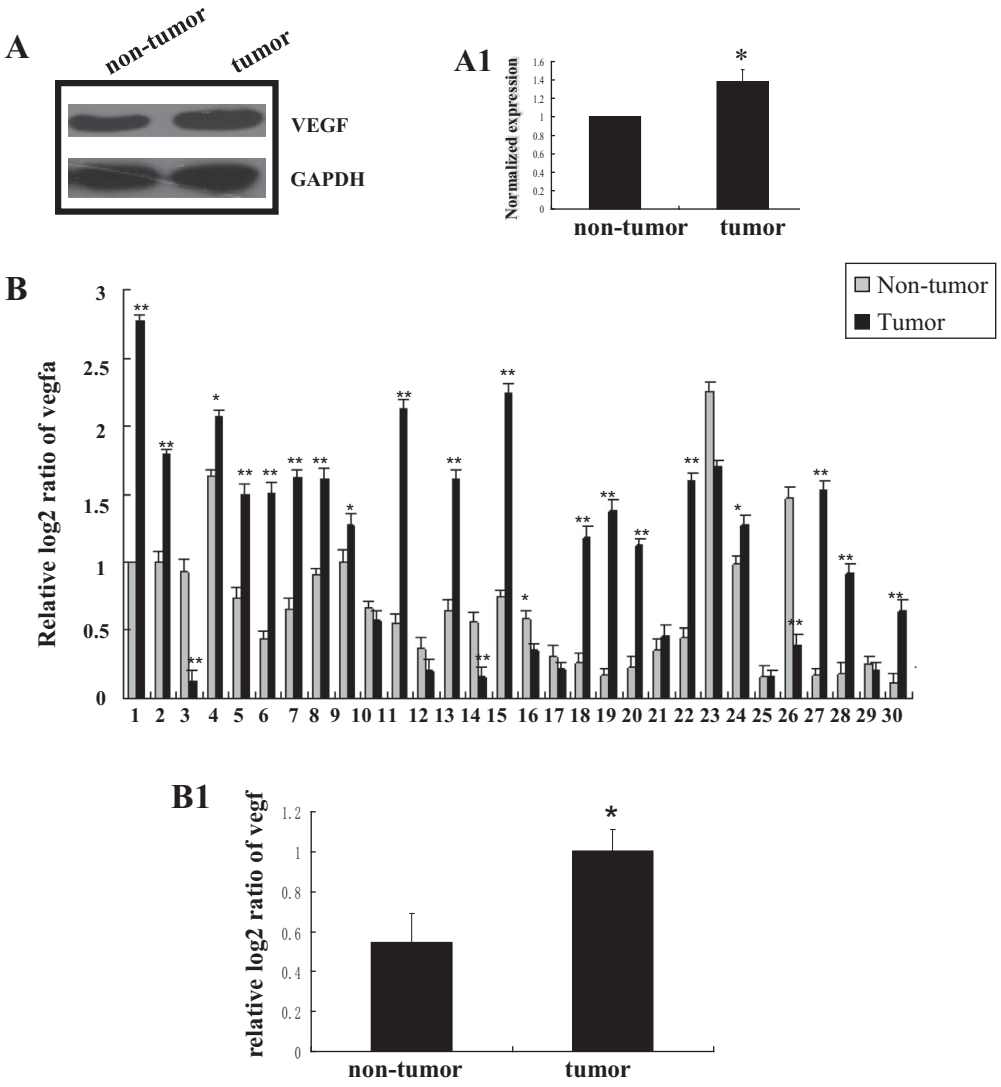

Fig. 7. Up-regulation of *Vegfa* in human breast cancer tissues. The cancer tissues from two patients with similar clinical parameters were pooled as a sample. (A) The protein level of VEGFA in human breast cancer tissues was detected by Western blot. The bands were analyzed using the Quantity One analyzing system (Bio-Rad). The expression of GAPDH is served as an internal control. The black histograms A1 represent the expression level of VEGFA in all breast cancer tissues. (B) The mRNA level of *Vegfa* in human breast cancer tissues and adjacent normal breast tissues was detected by qRT-PCR. Statistical analyses were performed to analyze the general trend of *Vegfa* in all human breast cancer tissues (B1). Gapdh is served as an internal reference. $^{*}P < 0.05$, $^{**}P < 0.01$.

ability of *miR-185* and 3′-UTR of *Vegfa*. Additionally, up-regulation of *miR-185* decreased the expression of *Vegfa* and down-regulation of *miR-185* increased the protein and mRNA level of *Vegfa* in vitro. A significant inverse correlation was also found between *miR-185* and *Vegfa* in human breast cancer tissues. Furthermore, the up-regulation of *Vegfa* could partially regain the overexpression of *miR-185*-mediated the suppression of cell growth and metastasis. The influence of Knockdown of *miR-185* on cell growth and metastasis was weakened down-regulation of *Vegfa*. Therefore, we confirm that *Vegfa* is the target gene of *miR-185* and *miR-185* may execute its tumor suppressor function partially by targeting *Vegfa*.

Correlations

		miR185	Vegfa
miR185	Pearson Correlation	1	-.573**
	Sig. (2-tailed)		.001
	N	30	30
Vegfa	Pearson Correlation	-.573**	1
	Sig. (2-tailed)	.001	
	N	30	30

**. Correlation is significant at the 0.01 level (2-tailed).

Fig. 8. The correlation between miR-185 and Vegfa in human breast cancer tissues. A linear correlation assay was carried out by SPSS16.0. $^{**}P < 0.01$.

Divertingly, our results indicated that *miR-185* level in breast cancer cells and breast cancer tissues was all oppositely related with the *Vegfa* mRNA level. Usually, miRNAs were supposed to exert function by inhibiting mRNA translation [20]. However, recent reports indicated that the alteration in protein level mediated by a miRNA were usually related with the changes of mRNA level, indicating that mRNA degradation might be a main element of mammalian miRNA suppression [21,22]. Our findings were also consistent with this reports.

Because a specific miRNA usually targets to multiple genes, *miR-185* excert its function may get through synergistic action of its target genes. In the future, besides *Vegfa*, other targets of *miR-185* still require to be confirmed in breast cancer. Additionally, animal experiment may be needed in order to further validate the function of *miR-185* and the relationship between *miR-185* and *Vegfa* in vivo.

In conclusion, *miR-185* is obviously diminished in human breast cancer tissues, which facilitates breast cancer cell proliferation, invasion and migration and suppresses cell apoptosis. *MiR-185* excerts these functions in breast cancer cell lines partly by regulating *Vegfa*. Our data will not only show the potential roles of *miR-185* in breast carcinogenesis, but also provide a clue for diagnosis and therapeutics of breast cancer.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.09.045.

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